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Professor Thomas G. Brady,
Biochemistry Department, University College, Cork.

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C O N T E N T S.

ABSTRACT.

1. Purification of adenosine deaminase from calf mucosa.

Batch absorption on a suspension of DEAE-cellulose was tested as a method for speeding up the purification of adenosine deaminase, but was found to be unsatisfactory because of large volumes required for elution and poor recovery.

Modified chromatography, which eliminates preliminary equilibration of the extract with buffer, and increases the rate of flow on the column has proved satisfactory, giving an almost 50% recovery of enzyme with a specific activity of 385 E.U. after two chromatograms, instead of three chromatograms required in previous purifications.

2. Purification of adenosine deaminase from cat lung.

Cat lung has been found to have a high concentration of adenosine deaminase. The enzyme was purified by aqueous extraction of the dried tissue, precipitation of inactive protein by acidification to pH 4.5, and precipitation of the deaminase by acetone followed by resolution in water and chromatography on Sephadex.

3. Molecular weight determinations on adenosine deaminase from calf mucosa have been performed by two methods:

(1) the approach to sedimentation equilibrium which gave a value of 38,000 $\pm$ 4,000 and (2) gel chromatography which gave a value of 35,000 $\pm$ 2,000.

4. Amino acid composition of adenosine deaminase from calf mucosa:

The amino acids tryptophan, cystine, serine and threonine, which are unstable to acid hydrolysis, were estimated by special methods. Their concentration in adenosine deaminase is reported.

5. By the Sanger dinitrophenylation technique, the amino acid found in highest concentration was aspartic acid. A method for the removal of free amino acids found in adenosine deaminase is described.

6. Ultraviolet difference spectra and spectrophotometric titration of tyrosyl residues in adenosine deaminase.

The ultra violet absorption spectrum of adenosine deaminase at neutral pH is modified either by acidification of alkalination. The character of the difference spectra produced by either treatment is similar and can, in part, be explained by the breaking of tyrosyl hydrogen bonds.

Spectrophotometric titration in acid solution reveal the presence of hydrogen bonding between the hydroxyl groups of tyrosine and the carboxylate group of dicarboxylic acid residues present in the protein. The number of tyrosine residues in the protein is 12. Spectrophotometric titration in alkaline solution shows that 7 of these residues ionise instantaneously and are, therefore, not hydrogen bonded. The 5 bound groups can be further subdivided into "weakly" bound and "strongly" bound.

7. Deaminases from different sources show differences in pattern when examined by a zymogram technique.

Studies on Mammalian Adenosine Deaminase.

I. Purification of adenosine deaminase from calf Mucosa.

During the current year, purification of adenosine deaminase from calf mucosa has been continued in order to provide sufficient material for structural and other studies. Various modifications of the procedure have been tested with the object of developing a reliable and standardized method.

Batch Adsorption:

Two 40 gram lots of mucosa purified to Step 3 (acetone purification), (1), were treated by batch adsorption. Extract I, after dialysis to remove acetone, was equilibrated against 0.001 N citrate buffer pH 6.0 for 24 hours with four changes of buffer. Extract II, after dialysis to remove acetone, was treated immediately with 10 g. of a thick suspension of DEAE-cellulose, which had been previously equilibrated with 0.001 N citrate buffer pH 6.0. It was stirred gently for 1 hour, after which the DEAE-cellulose was filtered on a coarse grained sintered glass filter, and washed with 0.001 N citrate buffer to remove all unabsorbed protein. The moist pad of DEAE-cellulose was then suspended in 0.1 N citrate buffer for elution.

Extract II (equilibrated) was treated in exactly the same manner for adsorption and elution.

Table I summarises the results obtained with the two extracts.

TABLE I

	Vol. (ml)	Protein mg/ml	e.u./mg	Total e.u.	% Enzyme Recovery
Equilibrated					
Extract I	41	6.4	110.0	28,864	100
Supernatant after adsorption	69	0.98	0.061	4.0	0.014
[The cellulose was then washed free of unadsorbed protein.]					
1st Eluate in 0.1 N citrate buffer	111	0.62	168.9	11,622	40.2
2nd Eluate	52	0.35	251.4	4,576	15.9
3rd Eluate	53	0.189	258.3	2,464	8.5
4th Eluate	47	0.15	180.0	1,269	4.4
Total:	263			19,931	69.1
Non-Equilibrated					
Extract II	44	7.9	82.5	28,688	100
Supernatant after adsorption	124	0.75	.006	0.59	0.002
[The cellulose was then washed free of unadsorbed protein.]					
1st Eluate in 0.1 N citrate buffer	113	0.7	152.8	12,091	42.2
2nd Eluate	55	0.417	233.7	5,115	17.8
3rd Eluate	51	0.22	250.0	2,652	9.2
4th Eluate	44	0.20	152.5	1,342	4.6
Total	263			21,200	73.9

From this table it can be seen that the enzyme is completely adsorbed on to the DEAE-cellulose from both extracts, so that equilibration of the extract with buffer is unnecessary.

In both cases, 70% approx. of the applied enzyme was recovered in the eluate but this required four extractions with 0.1 N citrate buffer, which resulted in a greatly increased volume in which the enzyme was dissolved. The twofold purification which was attained was satisfactory.

Further batch purifications were carried out on non-equilibrated protein extracts in attempts to increase the recovery which compares unfavourably with that obtained by chromatography and to reduce the volume of the eluate.

In one case a larger amount of mucosa 120 g. was used. The material purified to Step 3 had an activity of 80 E.U. and yielded after batch absorption a preparation with an activity of 240 E.U., but the recovery was only 65% and the volume of the eluate, in spite of all attempts to keep it to a minimum, was double that of the original extract.

Chromatography, which was used in previous purifications, yielded 80 - 90% recovery of enzyme and the volume of eluate was always less than that of the applied extract. The chief disadvantages of chromatography are the length of time required for equilibrating solutions prior to application and the time taken in applying the solution to the column. From the experience gained in the batch absorption studies it was felt that the chromatographic procedure could be shortened and the following purification of a 400 g. lot of mucosa shows this to be correct.

The results of this purification are summarized in Table II.

TABLE II

	Vol. (ml)	Protein (mg)	% Protein Recovery	Specific Activity	Total E.U.	% Enzyme Recovery
Aqueous Extract	11,080	400,000	100	---	871,009	100
Enz. equil. for 1st Chromato-gram	850	5,950	1.49	83.5	496,825	57.0
Enz. equil. for 2nd Chromato-gram	470	1,363	0.34	331.3	454,020	52.2
Enz. equil. for concen-tration	500	1,120	0.28	384.6	430,750	49.5

The mucosa powder was purified to Step 3 (1) in 10 lots of 40 g. After removal of acetone by dialysis against running tap water and elimination of traces of undissolved protein by centrifugation, the extracts were stored in the frozen state until all the mucosa had been purified to this stage. It is possible to treat two lots of 40 grams per day so that the complete operation including dialysis of the final extracts can be completed in five days. The extracts, after mixing, had a volume of 850 ml. The recovery of enzyme of 57% was lower than usual.

This aqueous extract was then applied to a 35 x 2 cm DEAE-cellulose column, previously equilibrated with 0.001 N citrate pH 5.8, at a rate of 3 ml per minute, which is three times faster than the usual rate. This operation took 5 hours and the subsequent washing with dilute citrate buffer to remove unadsorbed protein took 6 hours. Gradient elution was then begun and the enzyme was recovered in 90% yield in a volume of 470 ml and with a specific activity of 330, which represents a fourfold purification and the best purification obtained to date by a single chromatogram. The absence of equilibration

prior to applying the extract to the cellulose column, and the fast flow rate through the column, both of which result in a substantial saving of time, have also lead to a better yield and purification of the enzyme.

This material was chromatographed a second time and resulted in 1.12 grams of enzyme with a specific activity of 385 E.U. and in a yield of 49.5% of the original starting material.

The enzyme was not subjected to a third chromatogram because the specific activity had reached the maximum value which has been attained in recent purifications and there is now evidence from studies on Sephadex columns that the enzyme is accompanied by 10% of an inactive material of approximately twice its molecular weight, but which is very similar in other properties, which may be formed by the aggregation of two molecules of the enzyme giving an inactive dimer.

Further chromatography of this preparation was also undesirable because of a small contamination by bacteria which developed in spite of the special precautions, e.g. tyndallization of cellulose, taken to eliminate them. Contamination, which is now very much reduced, is still a danger, particularly when a large batch of enzyme is being prepared, and has to be constantly guarded against.

Before storage, the purified enzyme was concentrated and sterilized by filtration through a bacteriological filter.

II. Purification of Adenosine Deaminase from Cat Lung.

The survey of animal tissues for adenosine deaminase given in the Annual Technical Status Report No. 1, p. 14-17 (1962), showed that the highest concentration of deaminase in the animals examined was present in cat spleen and lung. As lung is the larger organ, it was decided to use it as starting material in an attempt to purify the enzyme.

60 grams of acetone-dried lung powder was prepared by homogenising the lungs of twelve cats. The homogenate was dehydrated by extracting twice with four times its weight of cold acetone. After most of the acetone had been removed by filtration, the powder was exposed to the air until free from acetone and then dried thoroughly in a vacuum desiccator before being stored in the cold.

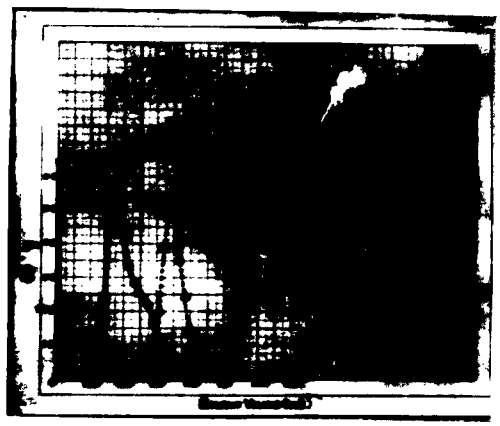
The steps which had proved successful in purifying adenosine deaminase from calf mucosa (1) water extraction, (2) acidification, (3) acetone fractionation and (4) chromatography, were tried.

Acid treatment of the aqueous extract to pH 4.5 achieved some purification by precipitating out protein devoid of activity, but it was not possible to achieve fractionation with acetone. Low concentrations of acetone produced only cloudiness in the solution without giving a precipitate. When precipitation did occur at 300 v/v% acetone the deaminase was precipitated together with all the other proteins. However, a considerable concentration of the enzyme was achieved as the deaminase could be dissolved in about 1/15 of the volume used for extracting it, and purification also occurred because some of the proteins were insoluble and were separated by centrifugation after overnight dialysis to remove the acetone. The solution was deep red in colour due to the presence of a chromoprotein, which is possibly haemoglobin.

Some purification was achieved by chromatography on DEAE-cellulose at pH 6.5 in 0.005 M phosphate buffer, and subsequent gradient elution. A quantity of unadsorbed protein with no activity was removed. On elution, a single protein peak was obtained. The absorption readings at 280 mμ, which measured the protein, and those at 405 mμ which measured the chromogenic material could be fitted on the same curve, indicating that the chief protein present was the chromoprotein. Adenosine deaminase activity was found in all regions of the protein peak and although the enzyme was about 55-fold purified, compared

with the original powder, it was still contaminated with chromoprotein, and twofold repetition of chromatography on the collected deaminase fractions failed to give a significant increase in specific activity. The highest specific activity achieved was 9.3 E.U.

A typical result of chromatography on a column of Sephadex G-75, equilibrated with 0.001 M citrate buffer of pH 5.8, is shown in Fig. 1.



O-O PROTEIN
Δ-Δ DEAMINASE

Fig. 1.

It can be seen that deaminase activity is eluted in a region low in protein at the terminal end of the protein peaks. The chromogen is associated with the region high in protein so that a considerable separation of this material from the deaminase has been achieved. In the Table below, the stages in the purification are summarized:

	Specific Activity	Yield %
Cat lung powder	0.28	100
Extract after acid treatment	1.66	95
Extract after acetone treatment	4.40	88
Eluate from DEAE-cellulose	9.30*	--
Eluate from Sephadex G-75	46.7	62

*From another experiment.

It can be seen from this Table that Sephadex chromatography produced a tenfold increase in activity, while chromatography on DEAE-cellulose only achieved a twofold increase. The overall purification using Sephadex was 166-fold, but as activity is not associated with a protein peak the deaminase is not yet pure and requires further fractionation.

III. Molecular weight determinations on Adenosine Deaminase from Calf Mucosa.

A series of ultracentrifugal studies were performed on a sample of the pure enzyme by Dr. E. Tully in the Department of Biochemistry, Brandeis University, Waltham, Mass., U.S.A. and the material gave a single symmetrical peak showing that the preparation was pure.

The sedimentation constant at a series of concentrations between 0.25% and 1% at pH 7.0 was measured. The results are given in the following table:

	Sobs.
10 mg/ml	3.152
10 mg/ml	3.248
10 mg/ml	3.199
7.5 mg/ml	3.150
5.0 mg/ml	3.215
2.5 mg/ml	3.212

It can be seen from this table that the sedimentation constant

does not alter with protein concentration, which is unusual as its value changes with concentration for most proteins.

This enzyme is rapidly inactivated at pH values below 3.0. An attempt was made to measure the sedimentation coefficient of acid inactivated enzyme in an effort to gain some insight into possible structural changes occurring on inactivation.

The enzyme was dissolved in 0.01 N HCl and adjusted to pH 2.0. It was held at this pH for 30 minutes and was then brought to pH 7.0 by addition of 0.1 N NaOH. When run in the ultracentrifuge it behaved completely different to the active enzyme. No value for the sedimentation coefficient could be obtained. An assymetric peak formed but collapsed rapidly. An interpretation of this behaviour has not been possible, but it is clear that the molecule has been radically altered by the acid treatment. The molecular weight determined by the approach to sedimentation equilibrium was found to be 38,000 $\pm$ 4,000.

Dr. P. Andrews of the National Institute for Research in Dairying, University of Reading, England, has kindly carried out molecular weight determinations on two separately purified samples of the deaminase, one sample was the same as that used by Dr. E. Tully. He carried out the determinations by his recently developed gel column chromatographic technique (2) using Sephadex G-100.

He found that the material used by Dr. Tully gave a single symmetrical peak for which a molecular weight of 35,000 $\pm$ 2,000 was calculated, which is in reasonable agreement with the value of 38,000 determined by the ultracentrifuge. The second sample had a main peak of molecular weight 35,000 but also contained a minor component with a molecular weight of 70,000 $\pm$ 5,000.

Studies carried out in this laboratory with Sephadex G-75 and G-100, using Dr. Andrew's technique, have also given this minor component of molecular weight 70,000. This material has no deaminase activity. The material present in the major peak

was now found to have a specific activity of 430 E.U.

When adenosine deaminase is inactivated by 8 M urea or 2 M guanidine and then examined by gel filtration, it was found that the peak corresponding to active material had completely disappeared. A single peak corresponding to material with a molecular weight of 70,000 was obtained. This result supports the view that the protein of molecular weight 70,000 is an inactive dimer of the enzyme.

IV. Amino Acid Composition.

Determination of concentration of (a) Tryptophan, (b) Cysteine, (c) Serine and threonine.

The amino acids listed above are destroyed to varying extents by the acid hydrolysis required to hydrolyse the peptide linkages in proteins, and so require special methods for their estimation.

(a) Tryptophan, which is completely destroyed by acid hydrolysis was estimated spectrophotometrically by the method of Goodwin and Morton (3), and after alkaline hydrolysis in the presence of starch by the method of Dreze (4).

The spectrophotometric method is performed on the intact protein after alkaline denaturation by measuring the absorption at wavelengths 280, 294.4, 340 and 370 mμ. Tyrosine, which also absorbs strongly in this region, can be estimated simultaneously. The results with adenosine deaminase were 1.63% tryptophan and 4.43% tyrosine.

Alkaline hydrolysis was performed by heating 5 mg of protein in the presence of 17 mg starch in 6 N $Ba(OH)_2$ for 15 hours at 120°C. The $Ba(OH)_2$ was removed as sulphate instead of carbonate to avoid losses of ammonia. The precipitate was removed by centrifugation and filtration and the supernatant solution was concentrated on a rotary evaporator. The concentrated solution was adjusted to pH 2.2 by addition of citrate buffer,

giving a final volume of 10 ml and 4 ml was applied to an 18 cm Stein and Moore Column for estimation. Preliminary experiments had shown that tryptophan comes out in a well separated peak before lysine. However, due to the high content of lysine relative to tryptophan in the protein, there was some overlapping of the tryptophan and lysine peaks. Tryptophan was, therefore, determined in different samples by (a) colour development with ninhydrin-hydrindantin solution and (b) by its absorption at 280 mμ.

Two separate hydrolysates with different batches of protein gave 1.68% and 1.57% by the ninhydrin colour development technique, while estimations at 280 mμ on the combined peaks in two further samples gave 1.56% and 1.76%.

The results by these three methods give very good agreement, as can be seen:

Spectrophotometry of intact protein	1.63%
After alkaline hydrolysis:	
(a) Colour development	1.63%
(b) Absorption at 280 mμ	1.64%

(b) Cysteine and cystine are destroyed to varying extents by acid hydrolysis, so that results on these amino acids are unreliable.

There is no evidence for the existence of free sulphydryl groups in this enzyme and it appeared from previous analysis that the level of cystine was low. An exact value for cystine was obtained by use of the method of Schramm, Moore and Bigwood (5), which converts the cystine to cysteic acid in the intact protein by performic acid oxidation. Cysteic acid is stable to acid hydrolysis and can be separated from other amino acids by chromatography on a column of Dowex resin 2 x 10 in the chloracetate form.

The protein after oxidation with performic acid was dried in a rotary evaporator and hydrolysed by refluxing with constant boiling HCl for 24 hours. As the cystine concentration of this

protein is low, it was necessary to use a quantity of hydrolysate equivalent to 10 mg of protein to get a reliable result. Portion of this hydrolysate was also chromatographed on the Stein & Moore column in order to have a direct comparison between the value for the cysteic acid and that of the other amino acids in this hydrolysate. The mean value for cysteic acid was 3.65%, which is equivalent to 2.64% anhydro 1/2 cystine.

(c) Serine and threonine, the two hydroxyamino acids, are destroyed at a constant rate by acid hydrolysis. The rate of their destruction was determined by hydrolysing the protein at 110°C for 24 hours and for 72 hours.

By applying the formula:

$$\log A_o = \frac{t_2}{t_2 - t_1} \cdot \log A_1 - \left(\frac{t_1}{t_2 - t_1}\right) \log A_2$$

where A_o, A_1, A_2 are the quantities of amino acids present at zero time and after time t_1 and t_2 the amount present in the intact protein can be calculated. The experimental values determined at 24 hours (t_1), 72 hours (t_2), and the calculated value at zer time are given in the following table.

	24 hours (t_1) %	72 hours (t_2) %	0 hour %
Threonine	4.95	3.39	5.55
Serine	3.62	1.73	6.24

V. End Group Analysis.

Previous studies have shown that when adenosine deaminase is dinitrophenylated and hydrolysed by the Sanger technique a number of DNP amino acids are liberated.

In the present investigation an attempt was made to determine their relative concentrations. This was particularly important for arginine, which being water soluble, in comparison to the others which were ether soluble, had not previously been

directly compared with them. The estimation of DNP-arginine was complicated by the presence of large amounts of DNP-E-lysine which is also water soluble and cannot be separated cleanly from DNP-arginine by paper chromatography. The following procedure was developed in this laboratory for the separation of DNP-E-lysine from DNP-arginine.

After dinitrophenylation and hydrolysis, the DNP-amino acids were absorbed on a talc column (6). The free amino acids, present in high concentration in the hydrolysate, are not absorbed by talc and pass through the column. The column was washed to remove all traces of free amino acids, after which the DNP-amino acids were eluted by a mixture of 4 parts ethanol + 1 part 1 N HCl. The eluate was then dinitrophenylated a second time. This converted DNP-E-lysine into DNP-dilysine, which is ether soluble. This solution of DNP-amino acids was extracted with ether in which all the DNP-amino acids except DNP-arginine are soluble. The DNP-arginine was adsorbed from the aqueous solution on a second talc column and was eluted in a concentrated solution. It was identified as arginine by the specific Sakaguchi test. Its concentration compared to DNP-aspartic acid present in the ether soluble fraction was low. The relative concentrations were 10 parts of DNP-aspartic to 1 of DNP-arginine, from which it can be concluded that it is very unlikely that arginine is an end-group.

The ether-soluble DNP-amino acids were separated by two dimensional chromatography according to Biserte and Osteux (7). However, DNP-aspartic and glutamic acids showed imperfect separation and were rechromatographed using the solvent mixture tert-amyl alcohol/potassium hydrogen phthalate pH 5 on Whatman No. 4 paper, which gave a very good separation of these two acids. The concentration of DNP-aspartic acid was found to be four times greater than that of glutamic acid. The results of this investigation indicate that there is a single N-terminal end group, aspartic acid, but this differs from our earlier findings

in which aspartic and glutamic acids appeared to be in almost equal concentration. Threonine, which has also been identified by chromatography in this and previous investigations, is present in such a low concentration, that it cannot be seriously considered as an N-terminal group. Serine, on the other hand, was present in half the concentration of aspartic acid.

(1) Removal of free amino acids from adenosine deaminase.

During the investigation of terminal amino acids it became evident that many preparations of adenosine deaminase contained small amounts of free amino acids which could not be removed by dialysis. Although the amount of these amino acids is low and would not affect the results in determinations of the total amino acid composition of the protein, their presence could affect the determination of end groups because, in this case, only a small number of amino acids are involved and larger amounts of protein of the order of 20 mg must be used.

It became essential to remove these amino acids which appear to be bound to the protein in some way because they cannot be removed by dialysis.

The first method studied was adsorption on a strongly acidic sulphonated cation exchange resin in the hydrogen form (Zeokarb 225). The protein solution was added to the column which is stated to retain amino acids but not protein. Quantitative recovery of protein was obtained but the eluted protein still contained amino acids when tested by precipitation with trichloroacetic acid (TCA) and examination of the supernatant by paper chromatography after concentration. This meant that amino acids were either not being fully adsorbed by the resin or TCA precipitation was itself causing a release of amino acids from the protein.

As there was a possibility that acidification alone might cause liberation of amino acids from the protein, precipitation by organic solvents was investigated. Alcohol, acetone and

mixtures of these solvents with ether failed to give quantitative precipitation of protein. However, an alcohol:chloroform mixture (3:1) proved satisfactory. On re-dissolving the protein and subsequent precipitation with TCA, a supernatant solution was obtained with no free amino acids. This method, therefore, provides a means of preparing protein completely free from amino acids. It also demonstrated that free amino acids were not liberated from the intact protein by acid treatment alone.

Determination of N-terminal amino groups with leucine amino-peptidase.

The interpretation of results obtained with leucine-amino-peptidase has been complicated by the presence of the free amino acids associated with the protein. While leucine -aminopeptidase certainly liberates amino acids from adenosine deaminase, it has not as yet been possible to identify the amino acid or amino acids which are liberated in highest concentration and which would, therefore, be the N-terminal group or groups. It is hoped that the elimination of free amino acids described in the previous paragraph will enable more definite conclusions to be drawn.

VI. Ultraviolet Difference spectra and spectrophotometric Titration of Tyrosyl residues in Adenosine Deaminase.

In recent years attempts have been made to correlate changes in protein structure resulting from denaturation, with ultra violet spectral changes, in the range of 230 to 320 mμ. Spectrophotometric titration based upon this phenomenon can be applied to the states of tyrosine residues present in protein molecules.

Crammer and Neuberger (8) first applied the method of spectrophotometric titration based on this principal to the investigation of the tyrosine residues in egg albumin and insulin. Similar investigations have since been performed on poly-L-tyrosine (9)

chymotrypsinogen (10, 16), trypsinogen (11), papain (12), lysozyme (13, 14, 19), bovine serum albumin (15, 16), angiotensin peptides (24), pepsin (17), insulin (18, 19) and ribonuclease, (20, 21). Inada (19) has recently demonstrated with catalase that 67 of the 93 tyrosine residues ionise instantaneously and that the remaining 26 residues are in some way bound in the protein molecule. He also found that of the 26 bound residues, 19 were "strongly" bound and that 7 were "weakly" bound in the protein.

The present investigation deals with similar studies on the states of tyrosine residues in adenosine deaminase in which three different types of residue have also been identified.

Materials:

Purified adenosine deaminase was used in each case, as prepared by the method of Brady & O'Connell (1). Preliminary results indicate a molecular weight between 35 - 45,000 and in this paper a figure of 40,000 has been used.

Protein was estimated by measuring the absorbancy at 280 mμ on a Beckmann-D.U. Spectrophotometer, with 10 mm quartz cells. The extinction was previously correlated with a total nitrogen estimation, which was determined by the Kjeldahl method.

The pH measurements were made at 18°C on a Radiometer Model 23 type pH meter, using a Radiometer glass electrode type G.200 B. All readings were corrected for sodium ion concentration with the aid of a nomograph supplied with the instrument.

Spectrophotometric Observations:

All spectra and difference spectra were recorded on a Perkin-Elmer Model 137 U.V. automatic spectrophotometer. Individual readings were taken on a Beckmann D.U. Spectrophotometer.

Spectral Changes in Acid and Alkaline Range:

To observe the spectral changes in the acid range, solutions of suitably diluted enzyme at acid pH were placed in the reference compartment, and a solution of the same enzyme concentration buffered at pH 6.0 was placed in the analytical compartment, and the difference in absorbance (E) between these solutions was recorded at 288 mμ.

Enzyme solutions adjusted to alkaline pH were read against the same concentration of enzyme buffered at pH 6.0 and the E recorded at 297 mμ.

The ionic strength of the acid and alkaline enzyme solutions was adjusted to $\mu = 0.1$ by addition of NaCl to bring them to the same value as the reference solution at pH 6.0. In all cases recordings were begun 15 seconds after pH adjustment.

Analysis of Results:

E values in this paper are calculated in terms of the number of tyrosyl hydroxyl groups per molecule of protein, assuming a molecular extinction for tyrosine of 2313, which is the value determined in the present study with tyrosine at 295 mμ.

Ultraviolet Absorption of Adenosine Deaminase.

The ultraviolet absorption spectrum of adenosine deaminase in acid, neutral and alkaline solution is shown in Fig. 2. As the spectrum in alkali alters with time, the curve shown for the alkaline solution can only be regarded as an approximation.

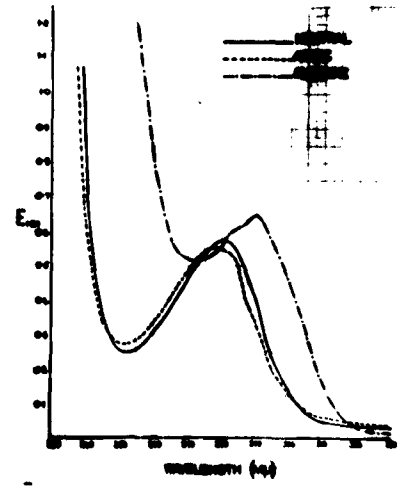


Fig 2

Ultraviolet Absorption in Acid Solution:

Since Crammer and Neuberger (8) postulated the theory of hydrogen bonding between tyrosyl residues and an acceptor group which might be a carboxylate ion, their views have been confirmed for many proteins.

An attempt is made here to ascertain if this theory holds true for adenosine deaminase by differential spectrophotometry as used by Laskowski (18) in studying insulin.

When adenosine deaminase at pH 1.1 and 2.5 respectively is compared in a spectrophotometer with a solution at pH 6.0, the curves 1 and 2 in Fig. 3 are obtained. The major peak at 237-238 mμ has been shown by Glazer et al. (16), working on bovine serum albumin, to be due to the change in the environment of the peptide backbone of proteins caused by denaturation.

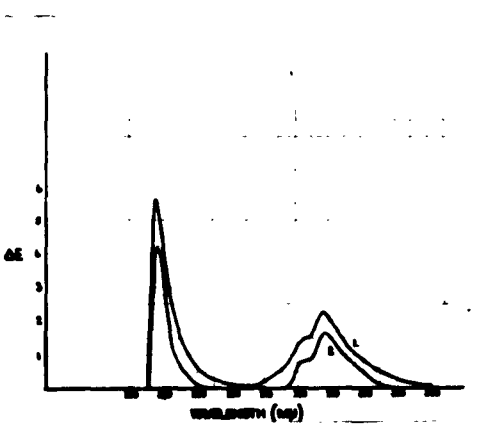


Fig. 3.

The peaks at 282 mμ and 288 mμ were also observed in insulin (22) and are characteristic of the shift in the tyrosyl spectrum towards longer wavelengths when the residue becomes hydrogen bonded in a protein.

The constancy of the ratio E/C in Table I shows that Beer's Law holds for this enzyme, from which it can be concluded

that the hydrogen bonding is intra- rather than intermolecular.

Table I.

mg/ml	E^{288}	E/C
0.571	0.107	0.187
0.844	0.152	0.180
1.100	0.201	0.182
1.600	0.296	0.185

To gain further insight into the significance of the changes in absorbancy at 288 mμ, shown in Fig. 3, a spectrophotometric titration between protein at pH 6.0 and at a series of acid pH values was performed. The results are shown in Fig. 4, from which an apparent pK value of 3.75 was calculated.

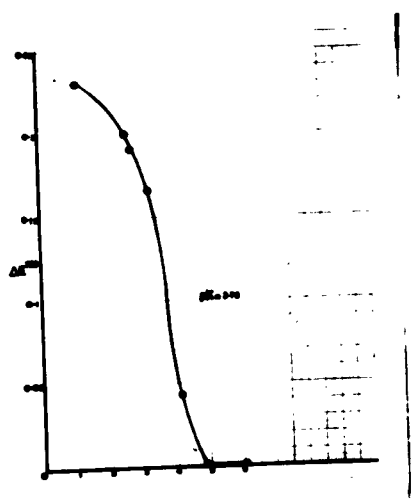


Fig. 4

Since in the pH range of 3 to 4.6 the carboxyl groups of dicarboxylic acids in proteins exist as carboxylate ions, the calculated pK value can be taken as an indication of the occurrence of bonds between such groups and the tyrosyl, as postulated by Laskowski (22) and Scheraga (23).

Ultraviolet Absorption in Alkaline Solution:

Fig. 5

Fig. 5 shows a difference spectrum between the enzymes at pH 6.0 and at pH 13. This curve is similar to that of tyrosine before and after ionisation of the phenolic hydroxyl groups. It appears that a measure of the amount of ionisation that occurs at any pH can be obtained from the molar extinction, either at 245 mμ or 297-298 mμ.

In the following experiments, the molar extinction was measured at 297 mμ, which allowed the use of more concentrated solutions of enzyme than would have been possible at the shorter wavelength.

It has previously been mentioned that the absorbancy of a solution of adenosine deaminase at pH 13 changes with time. On altering the pH from 6 to 13, there was an instantaneous change in molar extinction at 297 mμ from 10,100 to 32,500, followed by a slow increase giving a limiting value of 41,400 after approx. two hours.

To determine whether this time-dependent increase was due to the slow ionization of phenolic hydroxyl groups or to some other denaturation phenomena, a study of the change in absorption at the three wavelengths 265, 278 and 295 mμ was made. Tanford and Roberts (15) found that egg albumin gave no change at 278 mμ

a large change at 295 mμ and relatively small change at 265 mμ, while serum albumin showed the largest rise at 265 mμ, the smallest at 295 with 278 mμ intermediate. Crammer and Neuberger(8) had shown that the phenolic groups of egg albumin cannot be ionized without denaturation and that the ionization is irreversible from which Tanford and Roberts concluded that the spectral changes occurring in egg albumin were due to liberation of phenolic groups, while those occurring in serum albumin were due to some other denaturation process.

Fig. 6

It can be seen from Fig. 6 that with adenosine deaminase the spectral changes are similar to those found in egg albumin with maximum change at 295 mμ, no change at 278 mμ and an intermediate rise at 265 mμ, from which it can be concluded that the slow increase in molar extinction caused by alkali is due to the ionization of bound or buried phenolic hydroxyl groups.

Number of Tyrosyl Residues:

By comparing the E values at 295 mμ of a known tyrosine concentration at pH 13 with that of a known protein concentration at pH 13, the number of tyrosine residues per molecule of

deaminase was calculated to be 12.54, which is in close agreement with the value of 12 determined by chemical analysis, based on a molecular weight of 40,000.

Change in Molar Extinction:

The molar extinction at 297 mμ of solutions of deaminase in the pH range 5 to 8 is 10,085. This value was taken as the molar extinction of adenosine deaminase before ionisation of the phenolic hydroxyl groups. After complete ionisation of all phenolic groups at pH 13, the molar extinction approached the limiting value of 41,400. The total change in molar extinction at 297 mμ is 31,315, which corresponds to 2609 per tyrosine residue for 12 residues and is in good agreement with the value of 2,225 obtained for tyrosine at the same wavelength. Tanford and Roberts (15) found the change at 295 mμ per phenolic group to be 2430 for bovine serum albumin and a value of 2630 was obtained with ribonuclease.

The instantaneous change in molar extinction at 297 mμ is 22,408 which, on the basis of a change of 2,609 per tyrosine residue, means that there are 8.6 "free" tyrosine residues per molecule of adenosine deaminase.

Spectrophotometric Titration:

The nature of the slow ionization was investigated by measuring the change in absorption with time of a series of solutions at pH values between 8 and 13.

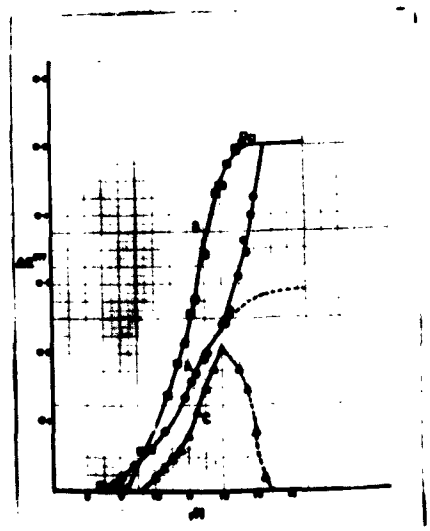


Fig 7

The values obtained immediately after mixing are shown in Fig. 7 curve A, while curve B gives the values obtained after the transformation had been completed. Curve A has an irregular shape and joins Curve B at approximately pH 13.3. Curve C, which is obtained by subtraction of Curve A from Curve B, represents the ionization of the "bound" residues. The height of Curve C at pH 12 indicates that 5 residues are of the "bound" type. This means that 7 residues are "free", which is somewhat lower than the figure of 8.6 calculated in the previous section. The apparent pK value determined from Curve C for the bound residues was 11.25, while a value of 10.8 for the free residues can be derived from Curve A.

Fig. 8

The "bound" residues can be further subdivided. Curve B, Fig. 8, shows the decay process of the fraction f of non-ionised forms observed at pH 12.2. It can be seen that the value of log f decreases rapidly at first, but then slows down and a linear relationship between log f and the reaction time is established. This occurs when all the "weakly bound" residues are ionised.

The extrapolation of the linear part in the later stage shows the time course of ionisation of the "strongly" bound residues, and its intersection at the ordinate gives the fraction of those residues to total bound residues in the molecule. The fraction of strongly bound residues thus determined for adenosine deaminase is four fifths of the total, which in this case is 4 to 1. This is the mean result of experiments carried out at seven different pH values between 10.75 and 13.0. If there are five bound residues in the molecule, this means that 4 are strongly bound and one is weakly bound.

The point on the curve in Fig. 8 where equilibrium was reached after ionisation of the weakly bound residues indicated the end point of ionisation of the "weakly" bound residues, and from this values of log f for the "weakly" bound residues were calculated, and plotted against time. The result is shown in Fig. 9.

Fig. 9

Using the formula of Imada (19), $\log f = -0.434 \times k(OH)^n t$, (where f is the fraction of non-ionised tyrosyl residues), the value of $0.434 \times k (OH)^n$ can be calculated as a function of

pH, from the inclination of the line as well as from similar data at other pH values. The linear relationship between log $k(OH)^n$ and pH in curve A, fig. 8, showed that the "weakly" bound residues ionise with the rate constant $k = 7.03\ M^{-1}Sec^{-1}$ and $n = 1$. This rapid ionisation with $n = 1$ indicates that these residues may be buried in the interior of the molecule and are not hydrogen bonded.

The pH dependency of log $k(OH)^n$ for the "strongly" bound residues which was calculated from the linear drop of log f in the later stage is shown by curve B, fig. 8, from which can be calculated that $k = 2.07 \times 10^2 M^{-1}Sec^{-1}$, and $n = 2$. The value of $n = 2$ indicates that the gradual ionisation was due to the presence of hydrogen bonding.

VII. Zone Electrophoresis.

Electrophoresis on starch gel combined with the zymogram technique reported in the Annual Technical Status Report No. 1, p. 14, 1962, has been used routinely in following enzyme purifications and in determining the number and type of isozymes present in different biological material.

Fig. 10

Fig. 10 is a photograph of a zymogram comparing adenosine deaminase in oat lung tissue with that of a mixed preparation of

calf mucosa. It can be seen that the enzyme preparation in cat lung is composed of two isozymes, while that present in the mixed calf mucosa consists of four isozymes. Furthermore, the isozymes from cat lung occupy different positions in the gel to those of the calf, which shows that they are different enzymes, although in some other properties they are similar.

The zymogram technique has also disclosed certain irregularities in pattern in the isozymes of calf mucosa. The results have not yet reached a stage when they can be reported but the investigation is being actively pursued.

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Implications of the Results for Future Work.

1. A convenient method for the purification of adenosine deaminase from calf mucosa in quantity and in good yield has been developed. This ensures an adequate supply of pure enzyme for further studies on the enzyme.

2. Progress in the purification of the enzyme from cat lung has been achieved. When it has been fully purified it will be compared with that of the calf.

3. The amino acid composition of mixed isozymes of calf mucosa, now completed, will be used for comparison with enzymes isolated from other sources and with the individual isozymes when a suitable method for their isolation has been perfected.

ANNEX.

Personnel employed on the Contract:

Professor T. G. Brady, Dr. E. Tully, Miss C.I. O'Donovan, M.Sc., M. O'Sullivan, M.Sc., J. Phelan, M.Sc., F. Hannigan, B.Sc. Technician - H. Keating.

The following estimate of the number of man-hours is based on a 44 hour working week, with 6 weeks per annum vacation, i.e. 46 working weeks or 2,000 man hours.

Five personnel worked 50% of their time on the project	5,000
Two personnel worked 100% of their time on the project	4,000
Total man hours	9,000

The amount expended on materials for the year 1st July 1962 to 1st July 1963 was £1,119.
